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13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have continued our studies of the immune mechanisms contributing to rejection of vascularized composite allografts (VCA) in murine models, and how these may be overcome to promote long-term allograft survival. We have now firmly established an orthotopic hind limb VCA model in our lab, and using this orthotopic model, have shown that either of 2 protocols, namely costimulation blockade (CD40L monoclonal antibody plus 2 weeks of rapamycin, RPM), or anti-TCR monoclonal antibody plus 2 weeks of RPM, can each achieve long-term VCA survival without maintenance immunosuppression. We are currently using these approaches to explore mechanistic details. Lastly, we have begun to test whether HDAC targeting may have effects on the VCA survival.

15. SUBJECT TERMS
allograft rejection, tolerance, costimulation blockade

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1. INTRODUCTION

The point of our study is to analyze the immune mechanisms contributing to rejection of vascularized composite allografts (VCA) using murine models, and to try and overcome these immune responses and promote long-term VCA survival.

2. KEYWORDS

Vascularized composite allografts, allograft rejection, tolerance, costimulation blockade

3. OVERALL PROJECT SUMMARY

Our goals for **CY16** were to develop Tasks 3 and 4; Task 3 is to test if peri-transplant immunotherapy will allow long-term VCA survival without development of chronic injury; Task 4 involves testing the ability of Treg-based therapies to promote VCA outcomes. Important progress on both Tasks 3 and 4 was achieved. Note, in the studies summarized below, at least 6 transplants/group (BALB/c->C57BL/6) were performed.

TASK 3

3.1 CD8+ T cell depletion does not promote VCA survival

Therapy with CD40L mAb (CD154, MR1)/donor splenocyte transfusion (DST) is thought to act primarily by immune modulatory effects on the CD4 T cell population, and so we questioned whether CD8 T cells might promote "breakthrough" rejection of limb VCA in this context. However, mAb depletion of CD8 T cells did not significantly alter the tempo of rejection in recipients treated with CD40L/DST, nor in recipients treated with RPM alone (**Fig. 1**). Hence, VCA rejection in our studies does not appear to involve a major role for CD8 T cells.

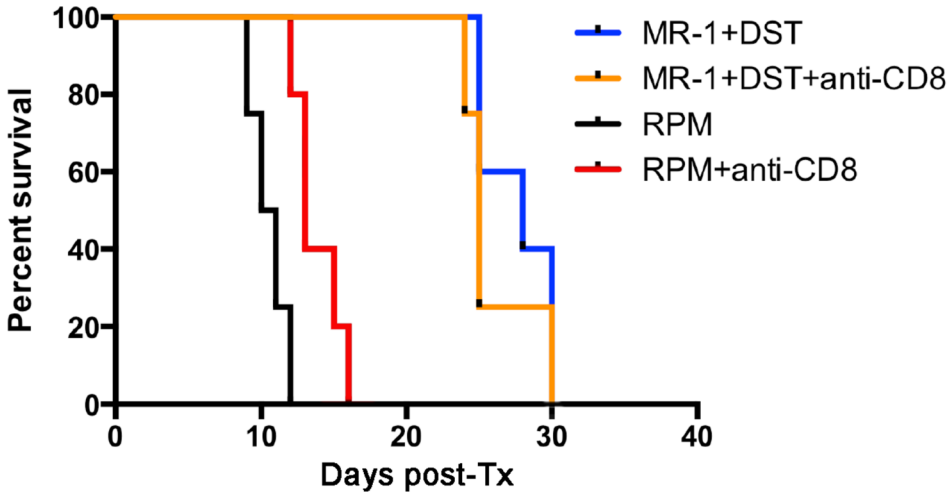

***Fig. 1**. CD8 T cells do not play a dominant role in VCA rejection in mice receiving costimulation blockade.*

3.2 CD154 plus DST and RPM promotes long-term VCA survival but not allograft tolerance

Even though CD154 mAb (MR-1) plus donor splenocyte transfusion (DST) is very effective at inducing tolerance of various allografts, such as those of hearts, kidneys or islets in mice, using fully MHC-disparate combinations such as BALB/c->C57BL/6 (1, 2), it was ineffective at prolonging corresponding hindlimb VCA survival. However, as noted in our previous reports, after RPM was added to the peri-transplant therapeutic protocol, long-term VCA survival was achieved (>100 days, $p<0.01$). This led us to test whether this long-term graft survival was accompanied by the development of donor specific tolerance by challenging recipients with third party (C3H) cardiac allografts. Surprisingly, long-surviving VCA recipients did not reject the third-party cardiac allografts, but instead rejected the original VCA tissues (**Fig. 2, left panel**).

To test whether RPM impaired tolerance induction by CD154/DST to non-VCA samples, we transplanted BALB/c cardiac allografts into C57BL/6 recipients and treated the recipients with either CD154/DST alone, or with RPM. One month later, the recipients were challenged with third party (C3H) hearts. As anticipated,

recipients treated with CD154/DST rejected the third-party C3H allografts but maintained their original BALB/c allografts. However, recipients treated with CD154/DST plus RPM did not reject their third-party allografts or their original cardiac allografts (**Fig. 2, right panel**). Hence, RPM erases the fundamental tolerogenicity of CD154/DST therapy. Analysis of blood samples within the early post-Tx period showed almost equal effectiveness of CD154/DST and CD154/DST/RPM protocols at inhibiting T cell alloresponses, with the exception that T cell proliferation (Ki-67+ CD4 and CD8 T cells) was higher in recipients that did not receive RPM therapy (**Fig. 3**).

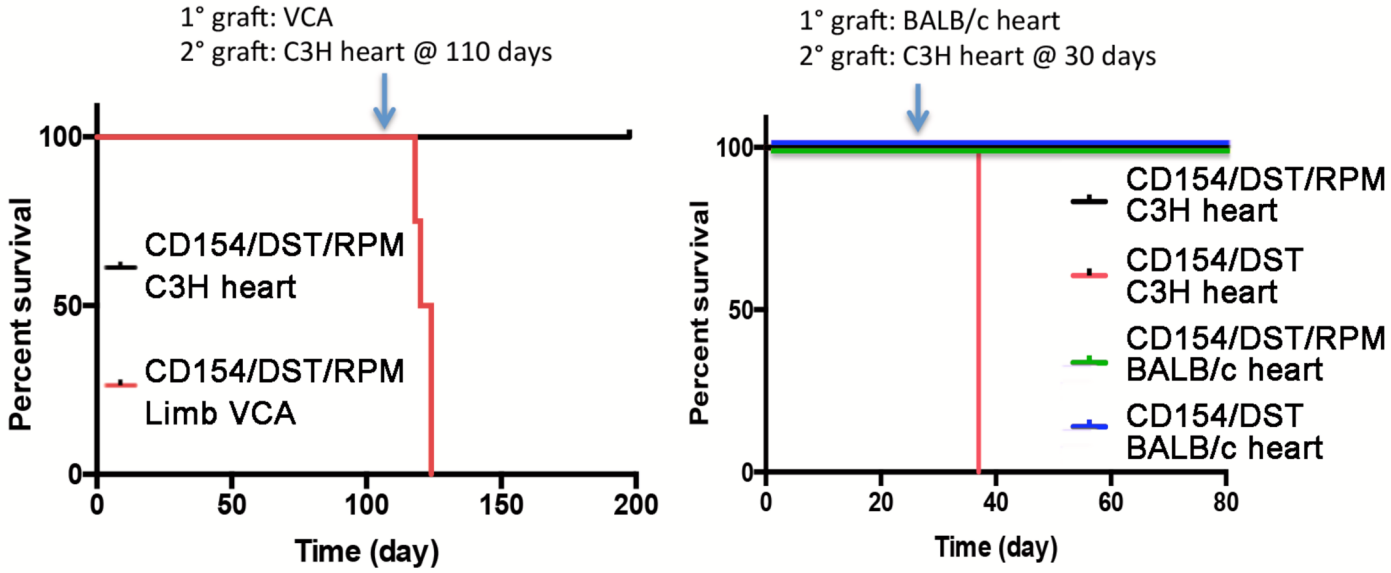

***Fig. 2**. RPM is necessary for long-term VCA survival but abrogates CD154/RPM-induced allograft tolerance.*

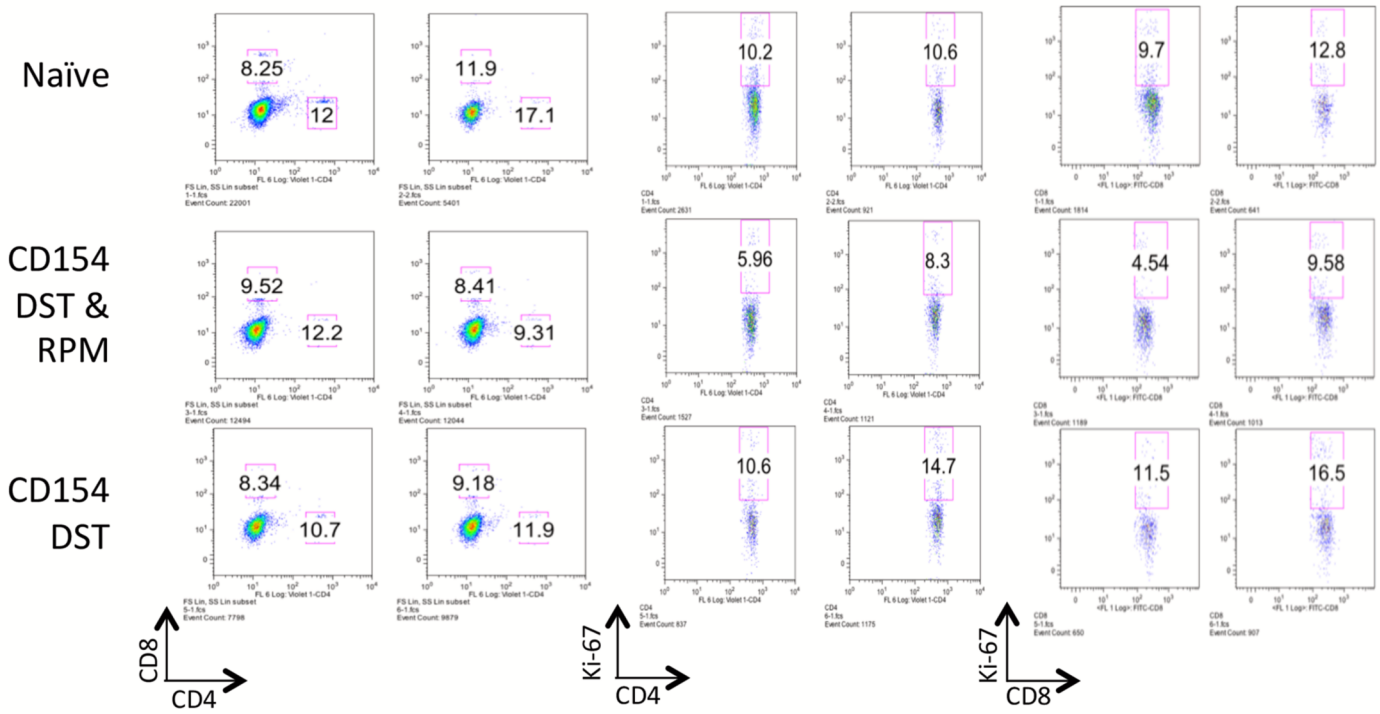

***Fig. 3.** Flow cytometry (in duplicate), at left, of CD4 and CD8 within spleens of normal mice (naïve), or VCA recipients treated as shown (day 7 post-Tx); central and right panels show corresponding CD4+Ki-67+ and CD8+Ki-67+ cells, respectively, in the same mice. Note how addition of RPM to the CD154/DST protocol decreases activation of host CD4 and CD8 cells (reflected by decreased Ki-67 expression).*

Collectively, these data (Figures 2 and 3) indicate that the addition of RPM to the CD154 mAb/DST protocol suppresses VCA-induced T cell activation, but also disrupts the development of donor-specific tolerance that normally accompanies such therapy. In the case of VCA, peri-transplant RPM therapy dampened immune

function to a useful extent, facilitating long-term VCA survival, but the rejection of VCA tissues as a result of third party cardiac allografting suggests the incomplete regulation of host alloresponses by Treg or other cells in the face of ongoing expression of skin-associated antigens (and is consistent with some clinical VCA data). By contrast, in the weaker cardiac graft model, in which long-term allograft survival and development of donor-specific tolerance was achieve using peri-Tx CD154 mAb/DST without RPM, addition of graft, consistent with anergy rather than Treg-dependent regulation, but this remains to be explored in more detail. We have previously shown that calcineurin inhibitor therapy completely abrogates the benefits of CD154/DST in allograft models (3). Taken together with the current data, immunosuppressive agents may be able to promote VCA long-term survival but cannot induce tolerance, and host alloresponses are decreased but by no means absent long-term.

3.3 CTLA4Ig plus DST and RPM also promotes long-term VCA survival

Various forms of CD154 and/or anti-CD40 mAb are in clinical development, but CD154 mAb is not clinically approved. In contrast, a second form of costimulation blockade (**COB**) involving use of CTLA4Ig ("Belatacept") to block CD28/B7 interactions is approved for use in human renal transplant recipients (4). With regard to potential direct clinical translation, we investigated if CTLA4Ig plus RPM might be effective, with or without concomitant DST, in a peri-Tx induction protocol. **Fig. 4** shows the results of testing CTLA4Ig (2 or 3 injections in the first week post-Tx and DST, CTLA4Ig and RPM (2 mg/kg/d, 28 d), or all 3 together. While CTLA4Ig plus RPM (purple) was able to achieve long-term orthotopic VCA survival, late rejection (>100 d) developed. By contrast, inclusion of DST (green line) in the protocol led to superior outcomes out to at least 150 days post-transplant. Hence, short-term COB with CTLA4Ig in the peri-Tx period can promote long-term VCA survival when accompanied by a brief sub-therapeutic course of RPM and one injection, post-Tx, of donor splenocytes. We have not yet tested if these long-term VCA recipients are tolerant (rejecting third party allografts), or whether, as with CD154 mAb/DST/RPM (section 3.2), use of RPM disrupts COB-induced tolerance induction.

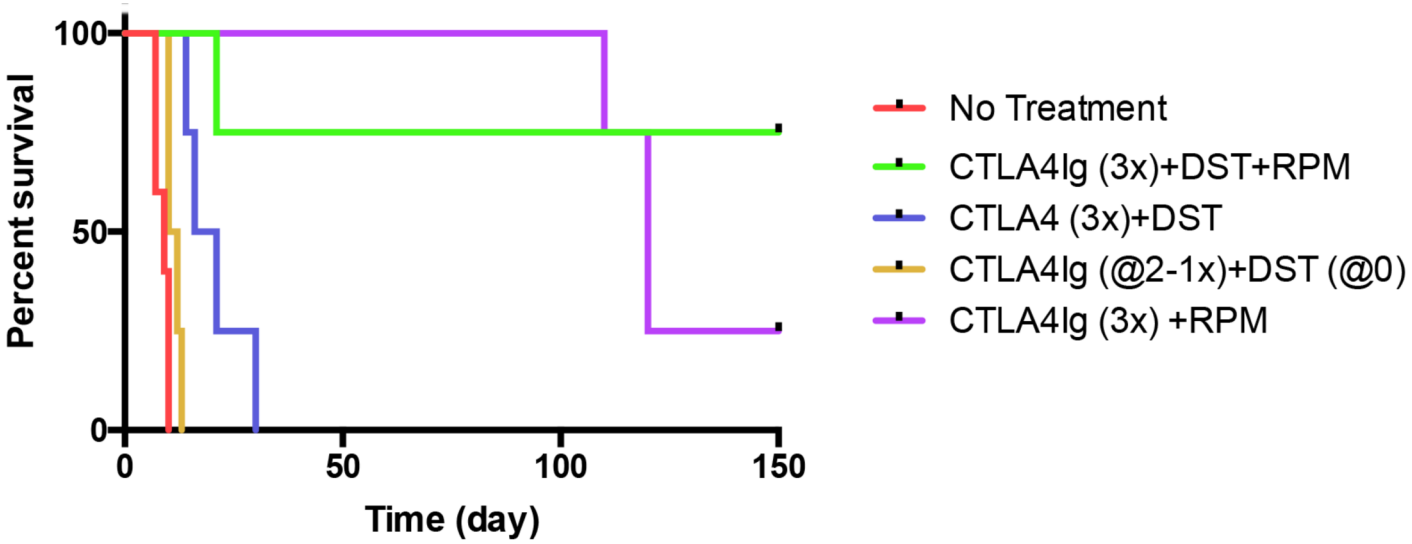

***Fig. 4**. Optimal orthotopic VCA survival using CTLA4Ig requires RPM and DST (p<0.01 vs. CTLA4Ig/RPM).*

3.4 Mechanisms underlying the efficacy of costimulation blockade (COB)

a. Efficacy of COB is radiation-sensitive

We achieve >100 days of VCA survival using 1 injection of CD154 mAb (MR-1) plus a 5 million donor splenocyte transfusion (DST), followed by 14 days of therapy with RPM (green, **Fig. 5**). However, the effects of CD154/DST/RPM are abrogated if BALB/c donor mice underwent whole-body irradiation (800 cGy) prior to their use as limb transplant donors (red line). Irradiation was not a non-specific cause of the graft injury since limbs from irradiated C57BL/6 (B6) mice were accepted long-term without any therapy (brown line, **Fig. 3**). Hence, these data point to the involvement of a radiation-sensitive component of the donor graft in facilitating long-term VCA survival in conjunction with costimulation blockade plus RPM.

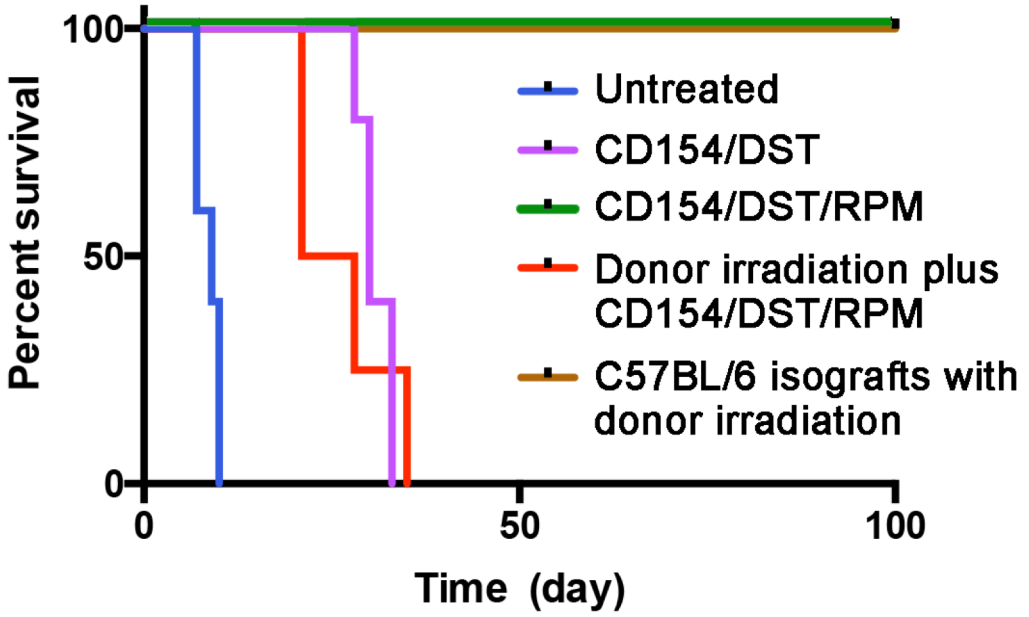

***Fig. 5**. A radiation-sensitive component of the donor limb is required for the efficacy of CD40L/DST/RPM; p<0.01 for irradiated group (red) vs. non-irradiated group (green) or irradiated isografts (brown).*

b. Long-term VCA survival cannot be restored by peripheral injection of BM cells

We reasoned that this dose of radiation might be affecting a bone marrow (BM) cell component required for long-term allograft survival using CD154/DST/RPM, such that peripheral injection of donor BM, at the time of transplantation, might restore efficacy of this protocol despite donor irradiation. However, as seen in **Fig. 6**, irradiated donor limbs were rejected despite use of costimulation blockade plus RPM (CD154 mAb/DST/RPM, shown in blue) or in conjunction with peripheral iv injection of 30 million donor BM cells (shown in green); the latter number approximates the numbers of BM cells that can be flushed from donor limbs pre-Tx. Hence, there is a radiation-sensitive component of the donor limb that is required for long-term VCA survival using CD154/DST/RPM, and this donor component cannot be restored by injection of donor BM cells in the periphery at the time of transplantation. These findings led us to further explore the basis for radiation-sensitive allograft survival in this context.

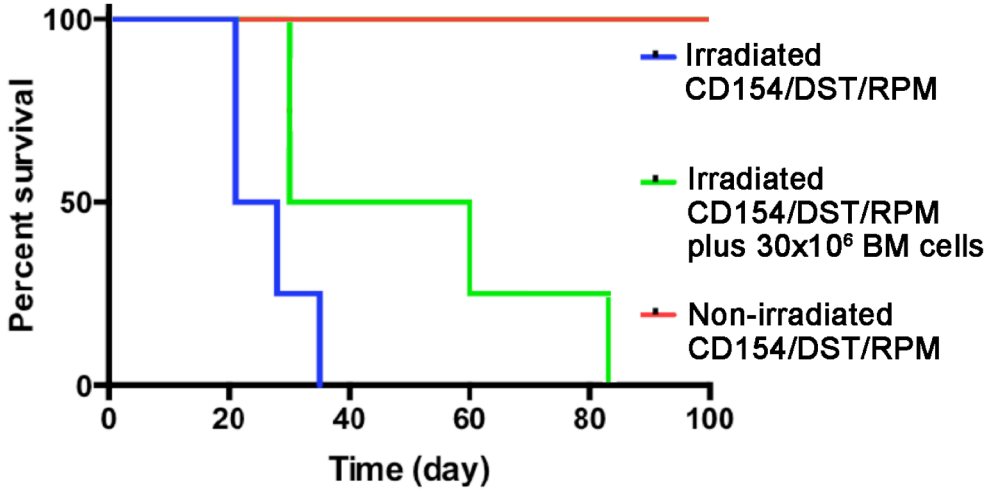

***Fig. 6**. The inhibitory effects of donor irradiation on VCA survival, despite recipient treatment with CD154/DST/RPM, cannot be overcome by intravenous injection of normal (non-irradiated) donor BM cells at the time or engraftment; p<0.01 for either irradiated group vs. non-irradiated group.*

c. Long-term VCA survival requires a bone-associated component but not donor T or B cells

In further exploration of which component of the donor limb was required for long-term VCA survival following CD154/DST/RPM therapy, we tested the effects of removal of the donor bone from the limb before engraftment. In contrast to the long-term (>100 days) survival of intact donor limbs engrafted under this protocol (blue line, **Fig. 7**), limbs lacking bone were rejected from 35-60 days post-Tx (green line, Fig. 7) (p<0.01 vs. intact limbs in mice treated with the same CD154/DST/RPM protocol).

Moreover, limbs from Rag1-/- mice, lacking T or B cells, were accepted long-term in recipients treated with CD154/DST/RPM (red line, Fig. 7).

• *Hence, the efficacy of this protocol requires donor bone, is radiation-sensitive, and must be present at the donor site, but does not require donor T or B cells.*

These findings led us to begin to examine events within the donor bone marrow following engraftment of BALB/c limbs into C57BL/6 mice treated with peri-Tx CD154/DST/RPM (COB/RPM).

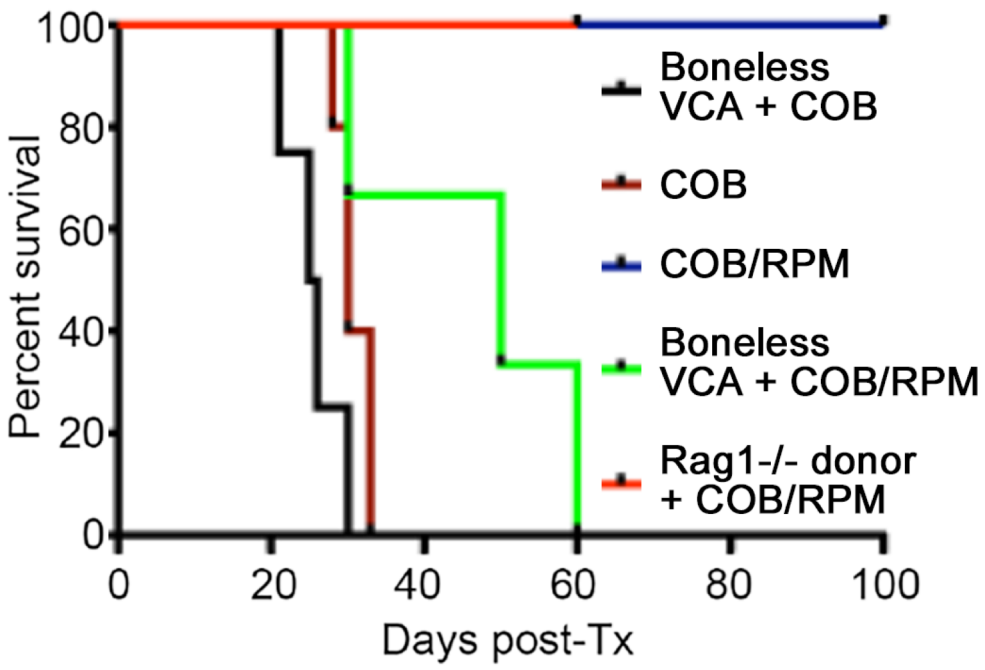

Fig. 7. Pre-Tx bone removal impairs VCA survival regardless of COB (black line) or COB/RPM (green) therapy, but an intact limb from an immunodeficient Rag1-/- donor does not affect long-term VCA survival using COB/RPM (red).

d. COB/RPM protects donor BM from rejection post-Tx and is associated with recipient Foxp3+ Treg infiltration

Analysis of cells within donor BM at 7 d post-Tx showed only small numbers of donor H-2K^b-negative cells in untreated controls, in contrast to large numbers of donor cells in recipients receiving COB/RPM (**Fig. 8**). Preservation of donor BM cells was associated with an influx of recipient CD4+Foxp3+ Treg cells (**Fig. 9**).

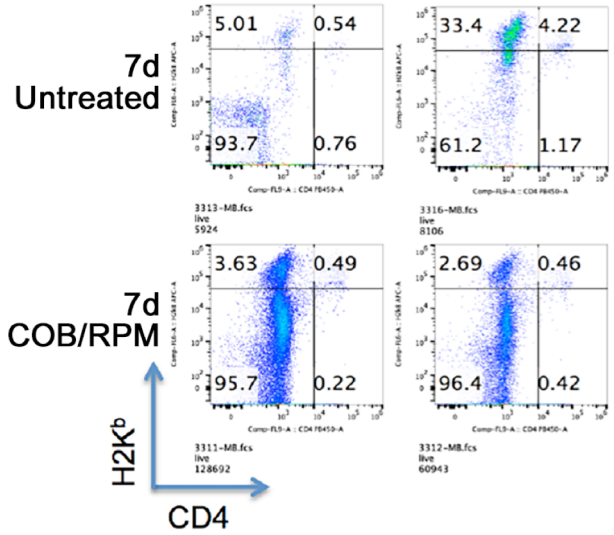

Fig. 8. Flow cytometry of duplicate samples of donor BM harvested at 7 d post-Tx from recipients that were either untreated or treated with COB/RPM. Comparison of bottom left quadrants in plots from the 2 groups shows paucity of H2K^b-negative cells (i.e. donor cells) in untreated mice, but preservation of large numbers of donor BM cells post-COB/RPM.

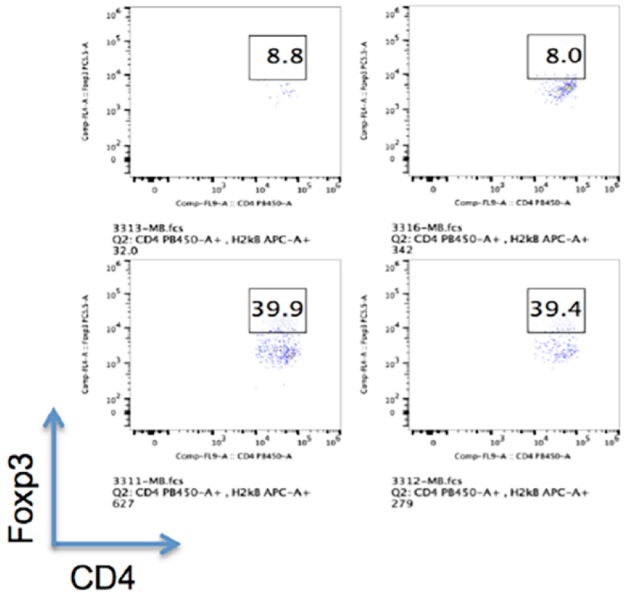

Fig. 9. Flow cytometry of duplicate samples of donor BM harvested at 7 d post-Tx from recipients that were either untreated or treated with COB/RPM. Compared to untreated mice, donor BM of mice receiving COB/RPM showed ~5-fold increased numbers of recipient H2K^b-positive CD4+Foxp3+ Treg cells.

The preservation of donor BM in VCA recipients treated with COB/RPM was also apparent when sections of donor grafts harvested at day 7 post-Tx were examined (**Fig. 10**). The marrow of untreated recipients showed widespread destruction of BM cells, whereas samples from mice receiving COB/ RPM showed preservation of BM cells, including leukocytes, erythroid cells and megakaryocytes.

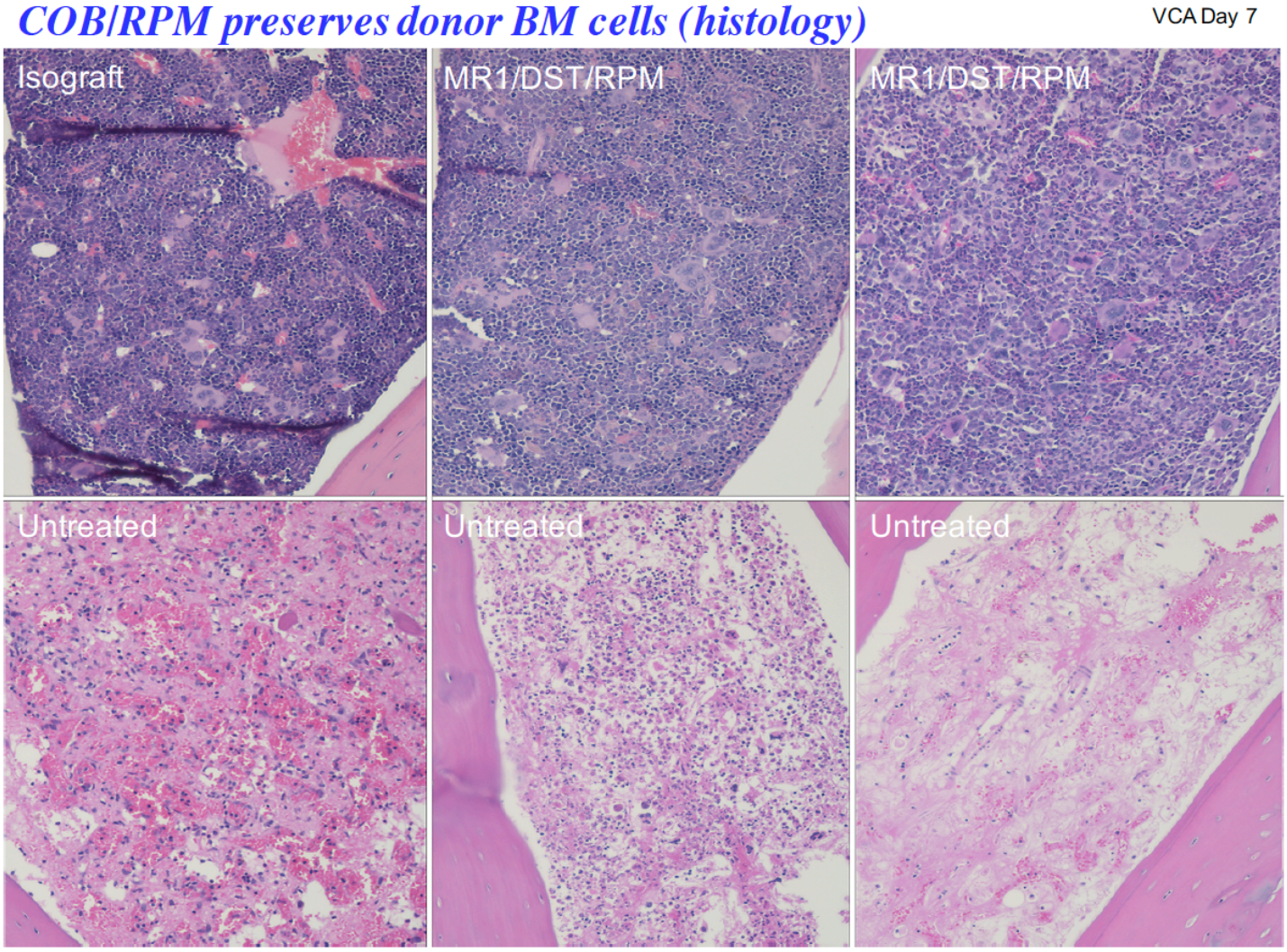

Fig. 10. Preservation of tri-lineage BM cells in association with COB/RPM therapy (H&E-stained sections of donor limbs at day 7 post-Tx).

e. Treatment of donors with a CXCR4 inhibitor disrupts long-term VCA engraftment post-COB/RPM

Most recently, we have found that the efficacy of COB/RPM is abrogated if donors are treated immediately pre-Tx with AMD3100, a CXCR4 inhibitor, that mobilizes BM cells (including hemopoietic stem cells and myeloid cells) (**Fig. 11**). We are currently pursuing further this line of investigation.

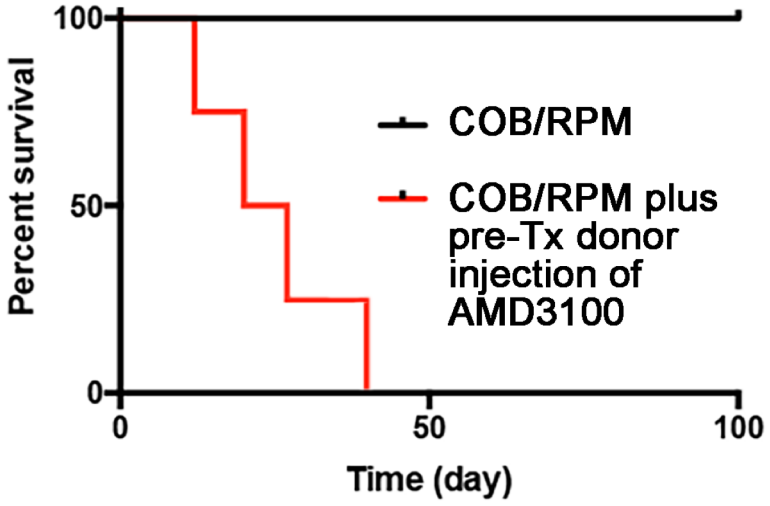

Fig. 11. Treatment of the donor with a CXCR4i immediately pre-Tx disrupts the effects of COB/RPM and leads to acute rejection.

TASK 4

Task 4 involves testing the ability of Treg-based therapies to promote VCA outcomes. We have set up a model to test aspects of Treg adoptive cell therapy. Immunodeficient (Rag1-/-) B6 mice are engrafted with BALB/c donor limbs and mice are reconstituted with 1 million recipient conventional T cells (T-effector, TE) cells ± varying ratios of B6 Treg cells. As seen in **Fig. 12**, use of TE cells alone led to rejection of 50% of grafts by 12-13 days post-Tx. The addition of 0.5 million Tregs did not significantly enhance VCA survival ($p>0.05$). In contrast, addition of 1 million Tregs (i.e. use of a 1:1 ratio to TE:Treg cells) prolonged VCA survival to about 40 days ($p<0.01$).

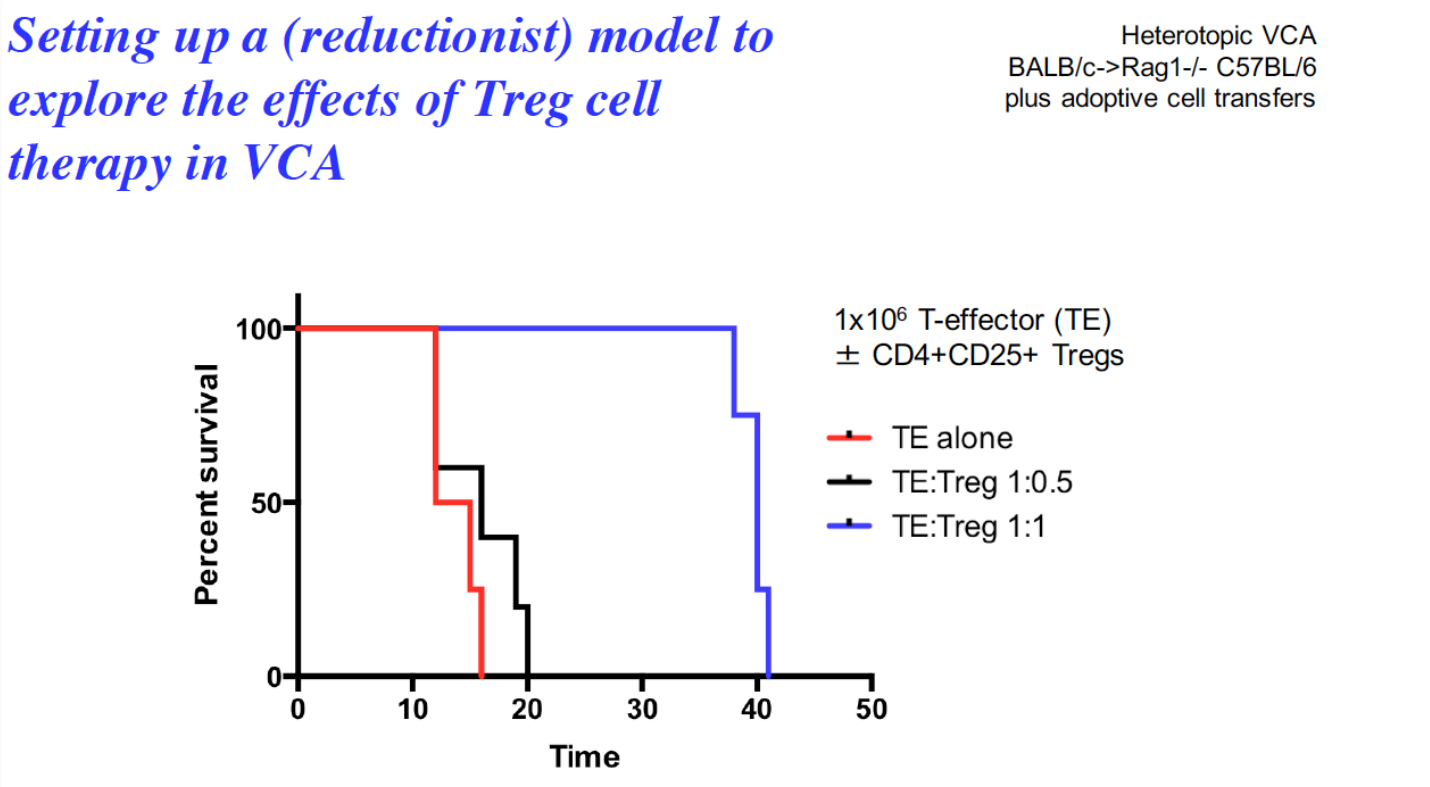

***Fig. 12**. Initial data using Treg adoptive transfers, as indicated in the Figure.*

Additional studies (data not shown) indicated that this beneficial effect of Tregs (1:1 ratio) was associated with preservation of donor (H-2K^d-positive) Foxp3+ Tregs as well (i.e. initially present within donor BM and thereafter able to be detected within recipient lymphoid tissues post-Tx).

4. KEY RESEARCH ACCOMPLISHMENTS

With regard to TASK 3:

• The efficacy of MR1/DST/RPM (COB/RPM) is abrogated by irradation of donor limbs, and depends on the presence of an undefined, non-lymphoid component of donor bone; i.e. COB still works if the donor limb is from a Rag1-/- donor mouse.

• Intravenous administration of donor BM cells does not restore the efficacy of MR1/DST/RPM in recipients of irradiated VCA; i.e. it appears that either marrow cells have to be administered within the donor tissue itself or that a non-marrow component is involved.

• Initial studies indicate that COB prevents early BM destruction post-VCA and leads to donor BM infiltration by recipient Foxp3+ Treg cells.

• Initial studies indicate that a component of the donor BM that can be mobilized by pre-Tx Rx of donors with AMD3100 (CXCR4i) is necessary for the efficacy of COB/RPM.

We propose to continue this work into the nature of the BM cells responsible, as well as to test pre-Tx treatment of recipient mice with FTY-720 to limit anti-donor responses post-Tx. Depending on the data we might thereafter focus on possible soluble factors (e.g. anti-CSF mAb Rx) ± imaging of intra-marrow events.

With regard to TASK 4:

• In reductionist, adoptive transfer models, though less efficacious than in cardiac allograft models using the same strain combination (BALB/c->C57BL6), Foxp3+ Treg cell therapy can have beneficial effects on VCA survival.

• Efficacy is associated with the persistence of donor cells (conventional CD4 and CD8 T cells, and donor Foxp3+ Tregs) in recipient lymphoid tissues, as well as by the presence of recipient Foxp3+ Treg cells.

These data suggest 2 broad lines of inquiry.

1. Prolongation studies

Effects of additional WT or HDAC-/- Treg infusions at serial intervals
Any added benefit of RPM therapy?
Any added benefit of HDACi therapy?

2. Mechanistic studies

Explore trafficking (imaging) of adoptively transferred recipient Tregs
Consider depleting transferred Tregs after initial period of VCA survival
Test effects of donor Treg infusion

5. CONCLUSIONS

Our long-term goal is improve the acceptability of limb and other forms of VCA as a therapeutic option in potential recipients. To that end, small animal studies using peri-transplant COB/RPM therapy are proving very encouraging. Interesting biology is arising concerning donor/host cell interactions, especially with regard to donor bone marrow. Is this a privileged or even tolerogenic site, and what are the mechanisms involved? Initial data indicate that Treg-based therapies can prolong VCA survival. This area is still largely unexplored, but a model has been established that, with the usual small animal caveats, can be used to test the effects of various cell therapy-based strategies and/or pharmacologic approaches.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Abstract

Cendales L, **Levine MH**, Bartlett S, Cheeseman J, Drachenberg C. **Hancock WW**, Joshi M, Kirk AD, Leopardi F, **Levin S**, Mehmet C, Selim A, Song M, Twaddell W, Wang L, Wang Z, Barth R.
Skin as a harbinger of rejection of underlying structures in VCA: Concordance or discordance?
American Transplant Congress
Boston, June 2016

Presentations

2 invited addresses by Dr. Hancock at international scientific meetings:

11/2015 "Progress in Experimental Vascularized Composite Allotransplantation"
14th Transplantation Basic Science Symposium
Lorne, Victoria, Australia

8/2016 "Immunology of VCA: Lessons from the Mouse"
26th International Congress of The Transplantation Society
Hong Kong

7. INVENTIONS, PATENTS AND LICENSES

None.

8. REPORTABLE OUTCOMES

None

9. OTHER ACHIEVEMENTS

None.

10. REFERENCES

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11. APPENDICES

Revised Quad Chart.

Positioning Vascularized Composite Allotransplantation in the Spectrum of Transplantation

CRMRP-JPC8, "Novel Immunomodulatory Therapies for Vascularized Composite Allotransplantation" MR120023P3

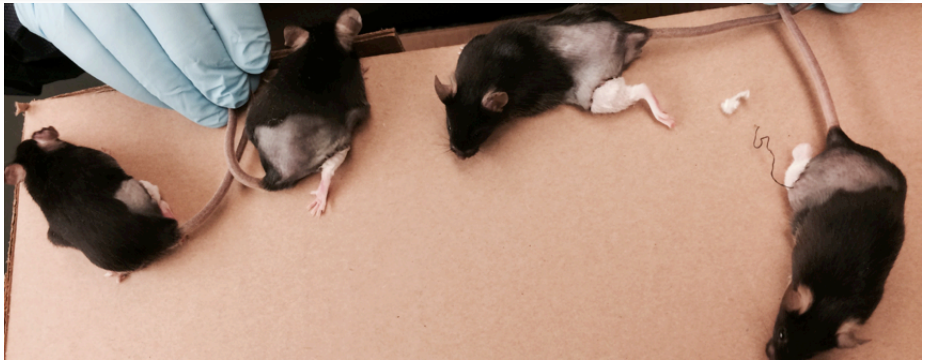
PI: Wayne W. Hancock Org: Children's Hospital of Philadelphia & University of Pennsylvania Award Amount: $1,996,875

Study Aims

- Establish murine hindlimb transplant models
- Target chemokine/chemokine receptor pathways promoting VCA rejection
- Test if costimulation blockade will promote long-term VCA survival
- Test if Foxp3+ Treg-directed therapies will promote long-term VCA survival
- Test optimal combinations of therapies so as achieve VCA engraftment and function, as well as preventing development of chronic injury

Approach

Our combined group recognizes that the long-term effects of chronic immunosuppressive therapies, including increased rates of nephrotoxicity, atherosclerotic disease, diabetes and tumor formation, outweigh their usefulness in VCA recipients. To identify less toxic and more suitable therapies for management of VCA, the group will undertake basic science studies in murine models to elucidate the mechanisms of immune rejection of VCA, and test the efficacy of novel strategies to achieve long-term engraftment without use of maintenance immunosuppressive therapy.



Examples of orthotopic hind-limb (stringent BALB/c->C57BL/6 model). • **Ultimately**, we wish to achieve long-term survival and function of orthotopic hind limb allografts in mice (BALB/c->C57BL/6). • **Accomplishment**: in our second year, we have achieved this for orthotopic hindlimb allografts (**photograph**), using peri-operative therapies that are less toxic than the current types of maintenance immuno-suppression used in organ transplant recipients.

Timeline and Total Costs (includes direct & indirect costs)

Activities	2013	2014	2015	2016
Task 1. Obtain regulatory approval and establish murine hindlimb models at CHOP				
Task 2. Target key chemokine/chemokine receptor pathways promoting VCA rejection.				
Task 3. Test if peri-transplant costimulatory blockade will allow long-term VCA survival without development of chronic injury.				
Task 4. Test ability of T-regulatory (Treg) based therapies to promote VCA outcomes.				
Task 5. Test optimal combinations of therapies based on data generated above.				
Estimated Budget (total $K)	511,875	495,000	495,000	495,000

Updated: October 14, 2016

Goals/Milestones

√**CY13 Goal** – We have established a VCA model and begun chemokine targeting (Task 1);
√**CY14 Goals** – We continuing chemokine/receptor targeting (Task 2), and using costimulation blockade, we have achieved considerable success (long-term engraftment with brief peri-Tx therapy);
√**CY15 Goal** - Complete costimulation blockade & Treg studies (Tasks 3 & 4)
CY16 Goal - Undertake final studies using optimal combinations (Task 5)

Comments/Challenges/Issues/Concerns

- If timelines change, comment here: no comments
- If off by more than 1 quarter, comment here; no comments

Budget Expenditure to Date

Projected Expenditure: As budgeted
Actual Expenditure: As budgeted